DEVELOPMENT OF *Salmonella typhi* Ty21a AS A POTENTIAL ORAL VACCINE AGAINST TUBERCULOSIS: SURFACE DISPLAY AND DNA VACCINE CARRIER OF A SYNTHETIC MULTI-EPITOPE MYCOBACTERIAL GENE

by

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This thesis is dedicated to my Parents and to my wife for her patient and encouragement and my children, Haya, Ahmad, Reema and Samar.

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List of Abbreviations

Amp	Ampicillin
AP	Alkaline phosphatase
bp	Base pair
BCG	Bacille Calmette-Güerin
BSA	Bovine serum albumin
DCIP	5-Bromo-4-chloro-3-indolylphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxy nucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FITC	Fluorescein isothiocyanate
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
MHC	Major histocompatapility complex
NTB	Nitroblue tetrazolium
OD	Optical density
PBS	Phosphate bufferd saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pfu DNA polymerase	*Pyrococcus furiousus* DNA polymerase
PE	Phycoerythrin
PMSF	Phenylmethylsulfonyl fluoride
PerCP	Peridinin chlorophyll protein
RNase	Ribonuclease
r-STVII	Recombinant *S. typhi* Ty21a
SDS	Sodium dodecyl sulphate
STVII-c	*S. typhi* Ty21a carries pJWVacII
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TB	Tuberculosis
TBE	Tris-Boric-EDTA
TE	Tris-EDTA
TSA	Tryptic soy agar
TSB	Transformation storage -buffer
TBS	Tris buffered saline
Ty21a	*S. typhi* Ty21a
TypJ	*S. typhi* Ty21a transformed with pJW4303
TypK	*S. typhi* Ty21a transformed with pKK223-3
U	Unit
UV	Ultraviolet
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosylpyranoside

Abstract

Despite the discovery of the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis*, more than 120 years ago TB remains a major worldwide health problem. Currently, the attenuated strain of *M. bovis*, Bacille Calmette-Güerin (BCG) is the only vaccine available against TB. Although BCG is the world's most widely used vaccine, its protective value as an anti-TB vaccine for adults in certain areas of the world, has been shown to be low or even non-existent. Thus there is general agreement that new novel vaccines are required for TB control and prevention especially in developing countries.

In this study, the use of the live attenuated typhoid vaccine, *S. typhi* Ty21a, for development as candidate vaccines against TB was explored in which the organism was utilized in a surface display system as well as a carrier of a DNA vaccine.

In the surface display approach, a surface display expression system was developed by the construction of a synthetic gene coding for the N-terminal of the ice nucleation protein (Inak-n) from *Pseudomonas syringae* using a method called assembly polymerase chain reaction (PCR). In this method, the Inak-n gene was assembled from 34 overlapping chemically synthesized oligonucleotides in a single step and amplified by PCR using specific cloning primers. The gene was cloned into the pCR®2.1-TOPO® vector to create a recombinant plasmid designated as pMSInak.

Cloning of a previously constructed 0.82kb synthetic gene known as VacII [which contained selected T cell epitopes of several *M. tuberculosis* genes namely ESAT6, MTP40, 38 kDa and MPT64 and further modified to include six consecutive histidine

(6xH) residues at the C-terminal end for affinity purification purposes] into pMSInak resulted in the fusion of the Inak-n and VacII genes and the resultant recombinant plasmid was designated as pMSInak-nVacII. The fused Inak-n::VacII (Inak-nVacII) gene from pMSInak-nVacII was then cloned into an expression vector pKK223-3 resulting in the final construct designated as pKMSInak-nVacII which when transformed into *S. typhi* Ty21a (creating the recombinant strain, r-STVII) and expressed allowed the fusion protein, Inak-nVacII, to be displayed on the surface of the host bacterial cells.

In the second approach, *S. typhi* Ty21a was utilized as a carrier of DNA vaccine. In this study, *S. typhi* Ty21a was transformed with a previously constructed DNA vaccine called pJWVacII to create a strain called STVII-c.

Both newly constructed vaccine candidates, r-STVII and STVII-c, were shown to be safe when tested in C57BL/6 mice. The immunogenicity of the two vaccine candidates in C57BL/6 mice were compared with each other and with the appropriate controls.

Each mouse was immunized orally with a dose of $2X10^9$ CFU of r-STVII or STVII-c (or controls) on Day 0 and Day 14 respectively and analyses were performed two weeks after the second immunization. The spleen cells of vaccinated mice were harvested and tested with the following assays: (i) Proliferation of T cells by thymidine uptake (ii) IFN-γ in spleen cell culture supernatant by ELISA and (iii) intracellular expression of IFN-γ by flow cytometry. In these studies, the purified recombinant protein (Inak-nVacII) and the synthetic peptides corresponding to single epitopes in the VacII protein were used as antigen specific stimulants.

The stimulation index of splenocytes from vaccinated mice with r-STVII was found to be about 2 fold higher than that of mice vaccinated with STVII-c. Conversely however, the concentration of IFN-γ secreted in the culture medium of splenocytes from mice vaccinated with STVII-c was 2 fold higher than that of r-STVII.

Intracellular cytokines analysis showed that both $CD4^+$ and $CD8^+$ T cells produced IFN-γ when splenocytes were stimulated in vitro with purified Inak-nVacII or the single epitope peptides. The data also showed that IFN-γ produced by $CD4^+$ T-cells from mice vaccinated with STVII-c was 1.3 fold higher than mice vaccinated with r-STVII when the cells were stimulated with purified Inak-nVacII. However, the data also showed that $CD8^+$ T-cells from mice vaccinated with STVII-c secreted 1.5 fold higher IFN-γ than mice vaccinated with r-STVII when stimulated with the same protein.

The importance of targeting both $CD4^+$ and $CD8^+$ T cells to stimulate effective protection against *M. tuberculosis* have been noted by many workers. In conclusion, the results obtained suggest that oral vaccination with the two new vaccine candidates produced in this study might be an efficient method for generating a broad and protective immune response against TB in the mouse model. The data generated by this study therefore may have an important impact in the strategy for developing newer vaccines against TB in humans.

PEMBANGUNAN *Salmonella typhi* Ty21a SEBAGAI VAKSIN ORAL YANG BERPOTENSI TERHADAP TUBERKULOSIS: KAEDAH PAMERAN PERMUKAAN DAN PEMBAWA VAKSIN DNA UNTUK GEN SINTETIK MULTIEPITOP MIKOBAKTERIA

Abstrak

Walaupun agen penyebab tuberkulosis (TB) iaitu *Mycobacterium tuberculosis* telah ditemui lebih daripada 120 tahun yang lalu, TB masih kekal sebagai antara masaalah kesihatan terbesar di dunia. Pada waktu ini strain *M. bovis* teratenuat, Bacille Calmette-Güerin (BCG) masih merupakan satu-satunya vaksin yang ada terhadap TB. Walaupun BCG merupakan vaksin yang paling tinggi kegunaannya di dunia, keberkesanan perlindungannya sebagai vaksin anti-TB untuk orang dewasa adalah rendah ataupun tiada lansung seperti yang ditunjukkan dalam kajian di beberapa tempat di dunia. Oleh itu adalah dipersetujui umum bahawa vaksin-vaksin baru perlu dibangunkan untuk membantu kawalan dan pencegahan TB terutamanya di negara-negara membangun.

Di dalam kajian ini, penggunaan vaksin hidup teratenuat untuk demam tifoid, *S. typhi* Ty21a, sebagai vaksin terhadap TB telah diterokai melalui penggunaannya dalam sistem pameran permukaan dan sebagai pembawa vaksin DNA.

Di dalam pendekatan pameran permukaan, sistem ekspresi permukaan telah disediakan melalui pembangunan gen sintetik yang mengkodkan terminal-N "ice nucleation protein", (Inak-n), daripada *Pseudomonas syringae* dengan menggunakan kaedah tindakbalas rantaian polimerase [polymerase chain reaction (PCR)] pemasangan. Melalui kaedah ini gen Inak-n telah dipasang dalam satu langkah dengan menggunakan 34 oligonukleotida sintetik bertindih yang kemudiannya di amplifikasikan melalui PCR

dengan menggunakan primer spesifik. Gen ini telah diklonkan kedalam vektor pCR®2.1-TOPO® untuk menghasilkan plasmid rekombinan pMSInak.

Pengklonan gen sintetik bersaiz 0.82kb bernama VacII (yang mengandungi epitop sel T terpilih dari gen-gen *M. tuberculosis* iaitu ESAT6, MTP40, 38 kDa dan MPT64 serta di modifikasikan untuk mengandungi 6 residu histidina di terminal C protein ini bagi tujuan penulenan afiniti) yang telah dibangunkan sebelum ini, kedalam pMSInak telah menghasilkan gabungan gen Inak-n dan VacII. Plasmid rekombinan yang dihasilkan di namai pMSInak-nVacII. Gen bergabung Inak-n::VacII (Inak-nVacII) daripada pMSInak-nVacII kemudiannya telah diklonkan ke dalam plasmid ekspresi pKK223-3 untuk menghasilkan plasmid rekombinan pKMSInak-nVacII. Plasmid ini apabila ditransformasikan ke dalam *S. typhi* Ty21a (dan menghasilkan strain r-STVII) dan diekspresikan akan menyebabkan protein bergabung ini, protein Inak-nVacII, dipamerkan di permukaan sel perumah ini.

Di dalam pendekatan kedua, *S. typhi* Ty21a telah digunakan sebagai pembawa vaksin DNA. Di dalam kajian ini *S. typhi* Ty21a telah ditransformasikan dengan vaksin DNA yang telah dibangunkan sebelum ini dan dinamai pJWVacII, untuk menghasilkan strain STVII-c.

Kedua-dua calon vaksin yang baru dibangunkan ini, r-STVII and STVII-c, didapati selamat bila diuji dalam mencit C57BL/6. Imunogenisiti kedua-dua calon vaksin ini dibandingkan di antara satu sama lain dan dengan kontrol-kontrol yang sesuai dalam mencit C57BL/6.

Setiap mencit imunisasikan secara oral dengan dos yang mengandungi $2X10^9$ CFU bakteria r-STVII atau STVII-c (atau kontrol) pada Hari Ke 1 dan Ke 14 dan analisis dijalankan 2 minggu selepas imunisasi ke dua. Sel spleen daripada mencit yang divaksinasikan telah dituai dan diuji dengan asai berikut: (i) Percambahan sel T melalui kaedah ambilnaik timidina (ii) IFN-γ dalam supernatan sel spleen melalui kaedah ELISA (iii) Ekspresi intrasel IFN-γ melalui flositometri. Dalam kajian-kajian ini protein rekombinan Inak-nVacII yang ditulenkan serta peptida-peptida sintetik yang mewakili epitop-epitop tunggal dalam protein VacII telah digunakan sebagai antigen spesifik perangsang.

Splenosit daripada mencit yang divaksinasikan dengan r-STVII di dapati mempunyai indeks stimulasi 2 kali ganda lebih tinggi daripada mencit yang divaksinasikan dengan STVII-c. Sebaliknya, kepekatan IFN-γ yang dirembeskan ke dalam medium kultur splenosit dari mencit yang divaksinasikan dengan STVII-c adalah 2 kali ganda lebih tinggi berbanding spelnosit daripada mencit yang divaksinasikan dengan r-STVII.

Analisis sitokin intrasel menunjukkan bahawa kedua-dua sel T $CD4^+$ dan $CD8^+$ menghasilkan IFN-γ apabila splenosit di ransangkan secara *in vitro* dengan Inak-nVacII yang ditulenkan ataupun peptida epitop tunggal. Data juga menunjukkan bahawa sel T $CD4^+$ daripada mencit yang divaksinasikan dengan STVII-c menghasilkan 1.3 ganda lebih banyak IFN-γ berbanding sel T $CD4^+$ daripada mencit yang divaksinasikan dengan r-STVII apabila diransangkan dengan protein Inak-nVacII yang ditulenkan. Walau bagaimanapun, sel T $CD8^+$ daripada mencit yang divaksinasikan dengan STVII-c

menghasilkan 1.5 ganda lebih banyak IFN-γ berbanding sel T $CD8^+$ daripada mencit yang divaksinasikan dengan r-STVII apabila diransangkan dengan protein yang sama.

Kepentingan mensasarkan kedua-dua sel T $CD4^+$ and $CD8^+$ untuk meransang perlindungan yang berkesan terhadap *M. tuberculosis* telah di nyatakan oleh ramai penyelidik. Sebagai rumusan, keputusan yang didapati mencadangkan bahawa vaksinasi oral dengan kedua-dua calon vaksin yang dihasilkan dalam kajian ini kemungkinan merupakan kaedah berkesan untuk menjana tindakbalas imun yang luas dan memberi perlindungan terhadap TB dalam model mencit. Oleh itu data yang dijanakan oleh kajian ini mempuyai impak yang besar terhadap strategi bagi membangunkan vaksin-vaksin baru terhadap TB dalam manusia.

Chapter One

Introduction

1.1. Background

Tuberculosis (TB) is an infectious disease caused by the tubercle bacillus, *Mycobacterium tuberculosis* which can attack different organs in the body, but most commonly the lungs. *M. tuberculosis* is a very serious human pathogen, and the World Health Organization have declared it among the leading fatal infectious diseases, as it remains the second leading killer infection after HIV/AIDS (Table 1.1). More than 3 million people die from TB (including 0.9 million HIV patients), and nearly 8 million new cases of this disease are reported each year (WHO, 2000, Sacksteder and Nacy, 2002). The vast majority of the reported TB cases and deaths occur in developing countries, due to poverty, rapid population growth, malnutrition, homelessness, crowded shelter, and lack of medical care. Since TB is easily transmissible between persons, the increase in TB in any sector of the population represents a risk to all sectors of the population (Dye *et al.*, 1999, Dye, 2000, Ainsa *et al.*, 2001). The global incidence rate of TB is growing at an annual rate of approximately 0.4% (WHO, 2003).

1.2. Human TB: A historical perspective

Medical historians suggest that TB is among the oldest infectious diseases that have affected humankind more than 5000 years ago. Tissue samples from Egyptian mummies grave sites, dated back to 3400 B.C., have shown evidence, either by morphological sign of TB, and/or by DNA analysis, that is consistent with an original *M. tuberculosis* complex similar to one that can be found today (Morse *et al.*, 1964, Crubezy *et al.*, 1998, Zink *et al.*, 2003). Around 460 B.C., Hippocrates described the

Table1. 1. Deaths from diseases for which vaccines are needed

Diseases	Deaths	%
AIDS	2,285,000	44.29
Tuberculosis	1,498,000	29.03
Malaria	1,110,000	21.15
Schistosomiasis	150,000	2.90
Leishmaniasis	42,000	0.81
Trympanosomiasis	40,000	0.77
Chagas disease	17,000	0.32
Dengue	15,000	0.29
Leprosy	2,000	0.03
Total deaths	**5,159,000**	**100.00**

Modified from: M. Kremer, Public Policies to Stimulate the Development of Vaccines and Drugs for the Neglected Diseases. CMH Working Paper Series Paper No. WG 2:8.

clinical features of both pulmonary and spinal TB: he wrote that TB was the most common disease of humans and can be transmitted from man to man, and he further noted that it was nearly always fatal.

TB has been known by many names such as Pthisis (Wasting), Pott's disease (TB of the bones), Lupus vulgaris (TB of the skin), Consumption (the "classic" case of lung disease), and White Plague. Tuberculosis-like diseases were reported in ancient writings of the Hindus and Chinese (Ayvazian, 1993, Daniel *et al.*, 1994). However, the first description of the transmissible nature of TB from a consumptive to healthy person was clearly established by the English physician Benjamin Martin in 1722. The control of TB was started in 1868, when a French military physician, Jean-Antoine Villemin proved that TB was contagious (Barnes, 2000). In Berlin on the 24th of March, 1882, Robert Koch announced the discovery of the TB bacillus after his success in growing them in culture. At that time, TB was very common and killed one out of every seven people living in the United States and Europe. Thus, this discovery was the most important step taken towards the control and elimination of this deadly disease (Barnes, 2000, Kaufmann, 2003). Nevertheless, in the 1900's, TB remained a common disease among the elderly people, and the only way suggested to lift the burden of the disease from the old people was to protect the future generations: infants, children, and the youth, before becoming infected. Thus, soon after the discovery of the tubercle bacilli by Robert Koch, the Sanatorium era began (Bloom and Murray, 1992).

Following these dates, TB declined in industrialized countries, as a result of the introduction of the effective vaccine Bacille Calmette-Güerin (BCG) in 1906, and the anti-tuberculosis drugs, streptomycin in 1944 and isoniazid in 1952, which cured

established disease, and prevented progression of TB infection to disease (Raviglione *et al.*, 1995, Maes, 1999). Consequently, there was a general decline in the attention to research in TB. However, hopes that the disease could be completely eliminated have declined since the rise of drug-resistant strains in the mid 1980s but this phenomenon have initiated renewed interest in the disease (Cole, 1994).

1.3. Epidemiology of TB in humans

In April 1993, the WHO took the exceptional step of declaring TB to be a global health emergency gaining attention to the problem that had been largely ignored over the preceding few decades (WHO, 1994). Since World War II until 1984, the incidence of the disease declined in Western Europe and North America due to anti-tuberculosis medications, awareness of the disease, and improved living conditions. TB has declined in USA from 84,304 reported cases in 1953 to 22,255 cases in 1984 (Fig.1.1), but the progressive decline in incidence stopped and the case level plateaued and then increased by 5% to 23,495 in 1989, and by 6% in 1990 (Groves, 1997).

Today, it is estimated that 2 billion people i.e., one third of the world's population are infected with *M. tuberculosis*. Over 30 million of those infected people harbour active disease. Every minute, more than 10 individuals develop TB amounting to 8 million new cases annually, and over 2 million of those TB sufferers are expected to die of the disease, making this disease the leading cause of death from a single pathogen in the world (Dye *et al.*, 1999). The incidence of TB has increased dramatically in areas with high rates of HIV infection.

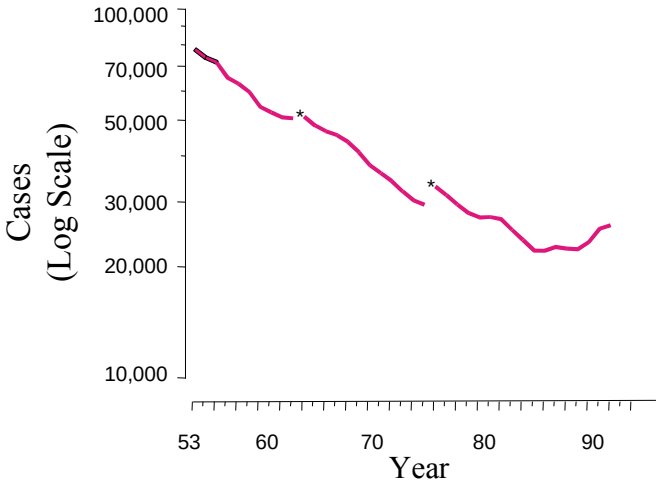

Fig.1. 1. Cases of TB reported to the Center for Disease Control and Prevention (CDC), in United States from 1953-1992. ⋆ Changes in case definition, data obtained from Groves(1997)

Thus, TB is the leading infectious cause of death among people more than 5 years of age in South-East Asia, and accounts for approximately 40% of all the cases of TB in the world. Within South-East Asia, more than 95% of cases are found in India, Indonesia, Bangladesh, Thailand, and Myanmar (Murray *et al.*, 1990, Kochi, 1991, Bloom and Murray, 1992). The Ministry of Health in Malaysia reported that 10,000-12,000 new cases were registered every year from 1972-1995. In the year 2000, WHO reported that 8,156 smear positive cases were notified in Malaysia (WHO, 2003).

Numerous factors have been associated with the reappearance and increased TB incidence which include: immigration from TB endemic areas, the emergence of multi-drug resistant (MDR) strains, and increased numbers of immunocompromised patients, especially HIV-infected. The above statistics put TB in the unfavorable list of the top major killers, together with AIDS and malaria (Kabra *et al.*, 2002).

1.4. The TB organism: *Mycobacterium tuberculosis*

Mycobacteria belong to the family Mycobacteriaceae and the order Actinomycetales. They are non-motile, non-spore forming, straight or slightly curved rod shaped microbes, 1-4 μm in length, and between 0.3-0.6 μm in diameter, making them smaller than most bacterial pathogens (Iseman, 2000). Mycobacteria are considered "acid-fast", which means that they retain dyes following an acid-alcohol decolorization step, and this characteristic is related to the complex cell wall structure that contains derivatives of mycolic acid (Floyd *et al.*, 1992). These organisms usually contain granules and vacuoles but they do not form capsules, flagella, or spores. In culture, these organisms grow slowly and divide once every 18 to 24 hours. They can be grown for 2 to 12 weeks, until they reach 10^3-10^4 in number (Dannenberg, 1992). They are resistant to

drying especially in sputum, where they can remain viable for 6-8 months. They are also resistant to 3% HCl and 6% H_2SO_4, and to 4% NaOH. However, Mycobacteria are sensitive to moist heat at 60ºC for 30 min, to disinfectants such as alcohol, glutaraldehyde, formaldehyde, and Ultraviolet (uv) irradiation (Tortora *et al.*, 2001).

Several species of mycobacteria with similar growth characteristics and biochemical reactions are classified together into the *M. tuberculosis* complex (Cole, 2002). In addition to *M. tuberculosis*, the complex includes *M. bovis*, *M. africanum*, and *M. microti* which are also causative agents of TB in mammals (Brosch *et al.*, 2000). *M. bovis* is the causal agent of bovines and infects a wide variety of mammalian species including humans. *M. africanum* has been reported to infect humans in sub-Saharan Africa as well as monkeys (Thorel, 1980). *M. microti* causes TB in small rodents such as voles (Hart and Sutherland, 1977).

Although the mycobacterial cell wall is weakly Gram-positive, this cell wall characteristic do not really indicate whether *M. tuberculosis* is more related to Gram-positive or Gram-negative bacteria since it has features of both in this respect. Recently Fu & Fu-Liu (2002) showed that *M. tuberculosis* is more related to gram-negative bacteria by construction of a genome tree based on the conserved gene content which revealed the evolutionary distance between nearest ancestral units.

1.5. Chemical composition of the *M. tuberculosis* cell wall structure

The cell walls of Gram-positive bacteria are made up of peptidoglycan layers combined with teichoic acid molecules, whereas those of Gram-negative bacteria contain much less peptidoglycan, with no teichoic acid (Fig.1.2). The Gram-negative cell wall has a

true lipid bilayer outer membrane that is attached to the characteristic endotoxic lipopolysaccharide (Sussman, 2002).

However, the cell wall structure of *M. tuberculosis* deserves special attention because it is unique among prokaryotes and may be a major determinant of the virulence of the bacterium. Biochemical and electron microscopic studies indicate that the cell wall of *M. tuberculosis* possesses four layers. The first layer (innermost) is the peptidoglycan layer while the next three surface layers are composed of lipids such as mycolic acid, glycolipids, cord factor and wax D (Sussman, 2002). The most important feature of the mycobacterial cell wall is the presence of up to 60% of the total mass of lipid components, particularly, the very long-chain mycolic acids, which are attached by ester bonds to the terminal arabinose units of the arabinogalactan, thereby forming a pseudolipid bilayer (Fig.1.2) (Brennan and Besra, 1997, Brennan, 2003).

In the cell wall of *M. tuberculosis* the lipids fall under two important classes, sulpholipids and trehalose dimycolates, which are also known as, cord factors. The sulpholipids are strongly acidic compounds covalently bound to trehalose sulphate. They may be involved in the virulence of *M. tuberculosis* as they have been shown to prevent phagosome/ lysosome fusion in macrophages infected with *M. tuberculosis*. Several waxes are also present, which increase the impermeability of the mycobacterial cell wall (Slots and Taubman, 1992).

This highly hydrophobic cell wall is not only responsible for the acid-fastness, but also for resistance to acidic or alkaline chemicals, and for its relative stability in simple disinfectants, in addition to the high adjuvanticity of the cell wall (Tortora *et al.*, 2001).

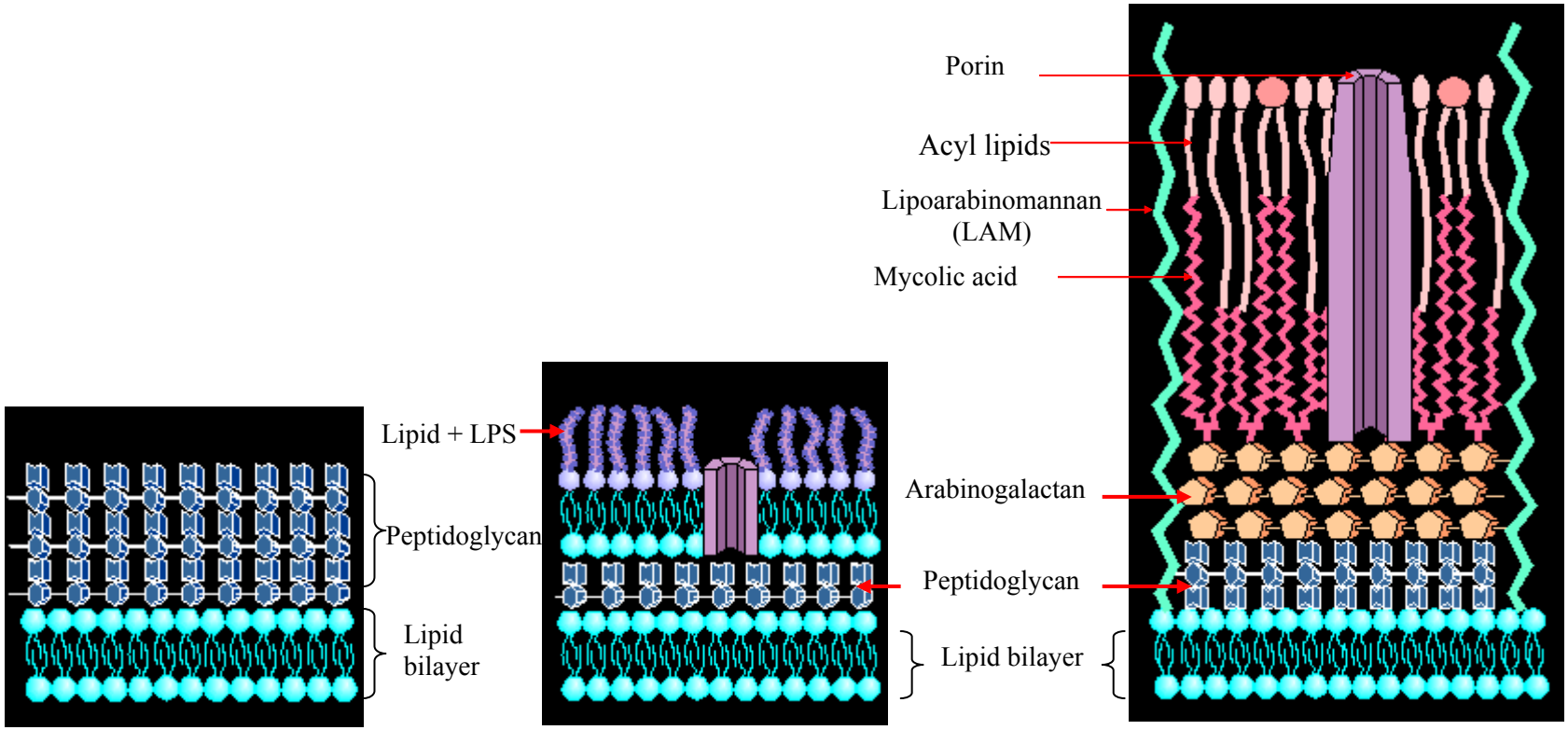

Fig.1. 2. Comparison between Gram-positive, Gram-negative and Mycobacterial cell wall.
Adapted from: http://web.uct.ac.za/depts/mmi/lsteyn/cellwall.html.

1.6. Genetics of *M. tuberculosis*

Genome sequencing of *M. tuberculosis* was completed in 1998 and analysis of the data show an estimate of 4,411,529 base pairs and 3,924 predicted open reading frames. *M. tuberculosis* is a difficult organism to study because of some unique features. One of these features is the high content of guanine and cytosine in its DNA. The high GC content of 65.6% may be one survival strategy employed by bacteria, since stability of DNA increases directly with number of GC bonds (Cole *et al.*, 1998).

1.7. Pathophysiology of TB

1.7.1. TB, the disease

Tuberculosis (TB) is defined as a pulmonary and systemic infectious disease caused by *M. tuberculosis* and characterized by formation of granulomas and by cell-mediated hypersensitivity, in which *M. tuberculosis* multiply and attack different parts of the body (Daniel *et al.*, 1994). The course of the disease is the result of a balance between the severity of the causative agent and the immunity of the host. Infection is encountered by inhalation of a droplet nuclei (1-5 μm in diameter) carrying the organism. Inside the body, the tubercle bacilli do not produce endotoxins or exotoxins (Edwards and Kirkpatrick, 1986, Dannenberg, 1992, Gonzalez-Juarrero *et al.*, 2001). Damage is caused by uncontrolled progressive, chronic inflammation and by the organisms living inside macrophages. TB is generally classified into latent infection and active infection.

In latent TB infection, *M. tuberculosis* is present in the body but there are no signs or symptoms of TB. People who have latent infection cannot spread the bacteria to other people but are at risk of developing active TB disease. Patients with active TB disease

exhibit signs and symptoms of disease. It occurs in 10% of those who are infected with *M. tuberculosis*. People who have active TB disease can spread the bacteria to others. TB can be categorized into two main types, according to where in the body the infection manifests itself. The two types are described as pulmonary and extrapulmonary (or non-pulmonary) TB. Pulmonary TB accounts for most of the cases of infection and about 85% of TB deaths (Rossman and MacGregor, 1995).

Extrapulmonary TB is a general term that encompasses TB infection that has disseminated to various sites around the body from the lungs. Extrapulmonary TB can affect many different parts of the body including lymphatic, pleural, spinal, bones and joints, meninges, genitourinary, peritoneal, and miliary (disseminated) TB. Pleural and lymphatic TB are the most common types of extrapulmonary TB (Fig 1.3). Extrapulmonary TB is very common among patients co-infected with HIV and pulmonary TB; 60-80% of these patients develop extrapulmonary TB in contrast to 17% of non-HIV patients who develop extrapulmonary TB. In addition, 30% of children with primary pulmonary TB infection develop extrapulmonary TB (Rossman and MacGregor, 1995).

1.7.2. Symptoms of TB

Most people infected with TB have inactive disease, which causes no symptoms. However, in primary pulmonary TB more than half of the people with this type of TB have no symptoms other than fever. In secondary or reactivation TB various symptoms are involved including cough, chest pain, night sweats, poor appetite and loss of weight. As the illness progresses, people may cough up blood (hemoptysis) and develop severe breathing problems (Mandell *et al.*, 1990).

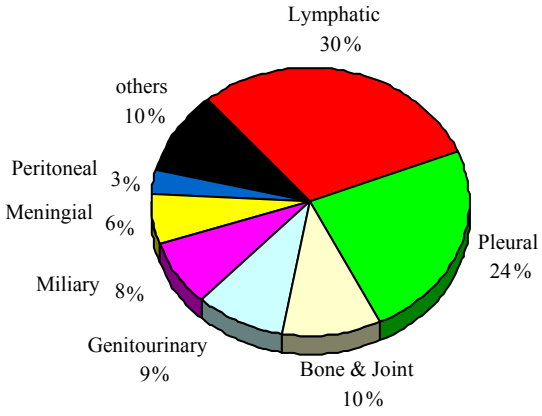

Fig.1. 3. Percentage of extrapulmonary TB by anatomic sites. (Rossman and MacGregor, 1995).

The symptoms of extrapulmonary TBs depend on where the TB has spread. Thus, if TB affects the lymph nodes, it can cause swollen glands, usually at the sides and base of the neck. In TB of the bones and joints, there can be a hunchback curvature of the spine or pain and swelling of a knee or hip, with patients usually developing a limp. Genitourinary TB, can cause pain in the side (between the ribs and hip), frequent and painful urination, with blood in the urine (Rossman and MacGregor, 1995).

1.7.3. Transmission of *M. tuberculosis*

Transmission of *M. tuberculosis* (infection) occurs when people with active pulmonary disease expel millions of water droplets containing *M. tuberculo*sis into the air. These droplets are small airborne particles of water that can remain floating in the air for several hours (Baum and Wolinsky, 1983). Every one may acquire *M. tuberculosi*s through these airborne particles regardless of age, sex and race, but not everyone exposed to the bacterium becomes infected nor does everybody develops clinical symptoms of TB. Studies conducted worldwide have shown a rapid spread of TB in crowded living conditions, such as in nursing homes, hospitals (Clarke and Higgins, 1995), homeless shelters, schools, military barracks, and prisons (Nettleman, 1993, MacIntyre *et al.*, 1999, Brewer *et al.*, 2001, Drobnieuski *et al.*, 2003). Passengers on commercial aircrafts are particularly susceptible for the transmission of TB among humans (Bignell, 1994, Miller *et al.*, 1996, Al-Jahdali *et al.*, 2003).

There are at least three factors influencing transmission of *M. tuberculosis*: (1) the number of viable bacilli in patient's sputum and their concentration in the air, (2) the length of time an exposed person breathes the contaminated air, and (3) the immune status of the exposed individual (Horsburgh, 1996).

1.8. Immune response to TB

1.8.1. Early response

Early after the initial infection with *M. tuberculosis*, granulocytes respond to this invasion and migrate from the blood into tissues and participate in an early inflammatory response. However, there is some controversy on the involvement of these cells in the killing of *M. tuberculosis* (Denis, 1991). *M. tuberculosis* induced an influx of leukocytes including polymorphonuclear neutrophils (PMNs), lymphocytes, and monocytes (Appelberg and Silva, 1989, Appelberg, 1992). The importance of neutrophils in host defenses against mycobacteria has been dismissed because the neutrophil has a short life span, and the bacilli grow inside macrophages, and are thus protected from the phagocytotic activity of neutrophils. These two reasons were supported by the findings of Denis (1991) who reported that human neutrophils were unable to kill *M. tuberculosis*. Furthermore, Pedrosa *et al*., (2000) showed in their study that *M. tuberculosis* is associated with or within macrophages and not with neutrophils. It has also been shown that eosinophils may be play a role in the inflammatory response initiated by *M. tuberculosis* (Castro *et al.*, 1991). These findings have led the researchers to focus their efforts on the *M. tuberculosis* inactivating mechanisms of macrophages instead of granulocytes.

1.8.2. Pathogenicity and initial defense against *M. tuberculosis* infection

1.8.2.1. Primary TB

Primary TB is a disease, or a response to infection by a host who have not been previously exposed to/or vaccinated against TB. After entry into the host, the droplet nuclei are carried down the bronchial tree and become implanted in the alveoli. The bacteria are ingested by the resident alveolar macrophages (AM) which kill or limit the

replication of mycobacteria, due to the action of lysosomal enzymes and reactive nitrogen and oxygen species (Fang, 1997, Miller and Britigan, 1997, van Crevel *et al.*, 2002). However, mycobacteria resist lysosomal degradation and escape from the phagolysosomes into the cytoplasm of the macrophage, where somehow, they manage to survive and even multiply until their number reaches 10^3-10^4 which is sufficient to elicit a cellular immune response (Smith and Wiengeshaus, 1989). Finally, mycobacteria burst out of the infected macrophage, killing it and then infect other macrophages in the area. As macrophages die by necrosis, they pour their lysosomal contents into the surrounding area or in the neighboring lung tissue, causing tissue damage, and initiating an inflammatory response. These events may be the most important stages in establishing infection in the host (Sompayrac, 1999).

Inflammation is necessary for the proper functioning of the host defenses, including the immune defenses, because it attracts circulating antimicrobial factors to the site of infection. These include phagocytes, lymphocytes, antibodies, complement and other antimicrobial components of plasma. The immune system continues to send macrophages to destroy the bacteria resulting in an accumulation of living and dead macrophages at the site of infection creating a structure called a tubercle (Dannenberg, 1989, van Crevel *et al.*, 2002). Two to three weeks after infection, cellular immunity developed, with antigen-specific T lymphocytes that proliferate within the early tubercles, and activated macrophages to kill the intracellular mycobacteria. As a result, most of the organisms die, and lesions in the lung and draining lymph nodes heal by fibrosis, and sometimes calcify to inhibit extracellular growth of the remaining mycobacteria. Some of these microorganisms remain viable for long periods (Daniel, 1994).

1.8.2.2. Secondary TB

The standard assumption of the recurrence of TB is by reactivation of the existing latent infection. However, reinfection by a new strain is also possible. Reactivation can occur when the immune system is weakened, as a result of malnutrition, and co-infection by other diseases such as AIDS (Chan *et al.*, 1996). When alveolar macrophages fail to kill the mycobacteria, the immune system's next line of defense is to form granulomas around the infected macrophages. Granulomas are, essentially, layers of T-cells sealing the mycobacteria inside a barrier from which it cannot escape. Two to three weeks after the inhalation of the *M. tuberculosis*, the host possesses both cell mediated immunity (CMI) and delayed-type hypersensitivity (DTH). With the emergence of DTH, infected macrophages in the interior of each granuloma are killed as the periphery becomes fibrotic and slowly become caseated (cheesy-like, semi-solid debris composed of lipid and proteins from tubercle bacilli and macrophages), and eventually merge into larger lesions (Dannenberg 1991). With time, proteases produced by activated macrophages liquefy the caseous material and form air filled tuberculous cavities that provide the bacilli with a suitable extracellular site for reproduction. Rupture of a tuberculous cavity into the pleural space may lead to the bacteria extending into the blood stream via regional nodes, where they are ingested by monocytes in the blood. Moreover, as the expanding lesion erodes through the wall of the bronchus, the liquefied content is discharged and thus allowing the bacteria to spread to and colonize virtually every organ in the host. A well aerated cavity is also formed where the organisms can actively proliferate (Dannenberg, 1991). Since inflammation of the surface of the bronchi causes increased mucus secretion and stimulation of the cough reflex, patients cough up sputum. Mycobacterial growth is not kept under control and can cause major destruction of tissues. In advanced TB, blood vessels may become

exposed to the cavities produced by necrosis, and patients may die of hemorrhage, if these vessels are ruptured (van Crevel *et al.*, 2002). Secondary TB usually becomes noticeable one or two years after the primary disease, probably because it takes that long a time to develop full blown delayed-type hypersensitivity.

1.8.3. Immunity to TB

1.8.3.1. Humoral immunity against TB

Humoral immunity is mediated by antibodies produced by B cells and their progeny when a foreign antigen enters the blood stream of a mammal. These antibodies bind specifically to antigens eliciting the immune response. The immune system then neutralizes or eliminates them from the body through ingestion and degradation of the antibody-antigen complex by phagocytes. One more method of elimination of foreign antigens is by degradation of viruses and other proteinaceous substances by proteolysis and killing of bacteria by cell lysis (Abbas and Lichtman, 2001).

Antibodies are produced in response to mycobacterial infection, but there is no definite evidence that immunoglobulins (Ig) play a significant role in protective immunity to TB (Dunlap and Briles, 1993). The role of the polymorphonuclear cells (PMNs) in TB is not well understood, but studies have shown that PMNs can diminish growth of *M. tuberculosis* by non-oxidative processes (Brown, 1987).

During the primary infection, IgM antibody responses are directed chiefly at nonspecific polysaccharide antigens. They develop early but never reach high titer, and this level does not correlate well with the presence or absence of active disease (Daniel and Debanne, 1987, Daniel *et al.*, 1994).

Levels of IgG antibody detectable by ELISA and other immunoassay are usually an indication of active TB. Studies with many TB antigens using several techniques showed that few control subjects have measurable IgG antibody levels (Daniel and Debanne, 1987).

IgA antibodies have been found at low levels in the serum of patients with active TB, but not in control subjects (Daniel and Debanne, 1987). Recently, Cardona, *et al*., (2002) showed for the first time the stimulation of antibodies against the glycolipids from the *M. tuberculosis* cell wall which include diacyltrehaloses (DAT) and sulpholipid I (SL-I) in murine models. Their results showed that these antigens elicit higher antibody levels than protein antigens like the Ag85 complex, culture filtrate proteins (CFP) and purified protein derivative (PPD).

The results from the studies described above suggest that although humoral immune system is induced by *M. tuberculosis* infection, it appears to play little role in protecting the host from the disease progression.

1.8.3.2. Cellular immunity against TB

Cell-mediated immunity (CMI) response is believed to involve different T-cell subsets. T-cells can be divided into two major classes, $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ and $CD8^+$ T-cell recognition of antigens requires that the antigens are processed and displayed on the surface of antigen presenting cells (APC) bound to specialized molecules called major histocompatibility (MHC) molecules. MHC molecules include class I and class II molecules (Germain, 1999).

One feature characterizing the class I MHC molecules is that they typically present peptides derived from intracellular antigens such as endogenous proteins or viral proteins synthesized during infection, and they are expressed on all nucleated cells. Peptide-MHC-I complexes are recognized by $CD8^+$ T-cells. These cells have cytolytic activity and will kill the target cells upon peptide-MHC recognition. They also have cytolytic functions and are generally called cytotoxic T lymphocytes (CTL). When a CTL recognizes its target, it releases cytotoxic molecules, perforins which will induce apoptosis in the target cells (Abbas and Lichtman, 2001, Esser *et al.*, 2003).

Class II MHC molecules are expressed by APC only and typically present peptides derived from extracellular antigens that have been internalized by the APC. The peptide-MHC complexes are mainly recognized by $CD4^+$ T-cells. $CD4^+$ T-cells can be further divided into two subsets of effector $CD4^+$ T lymphocytes, called T helper type 1 (Th1) and 2 (Th2) respectively, which differ in their cytokine production (Abbas and Lichtman, 2001, Esser *et al.*, 2003). Th1 cells are important in infections with intracellular bacteria such as *M. tuberculosis* and they produce interferon gamma (IFN-γ), interleukine-12 (IL-12) and tumor necrosis factor (TNF)-α. On the other hand, Th2 cells are important in antibody-mediated (humoral) immunity. These cells produce cytokines such as IL-4, IL-5, IL-6 and IL-10 that provide the necessary signals to B cells in producing antibody (Abbas and Lichtman, 2001, Esser *et al.*, 2003).

Cell-mediated immunity is the major protective immune response against mycobacterial infection. T-cells contribute in the acquired immune response by means of two major functions. First, they produce cytokines, particularly IFN-γ which is the major activator of antimicrobial macrophage functions. This cytokine is produced by all T stimulated

cells in response to mycobacterial infection, such as $CD4^+$ and $CD8^+$ T-cell clones (Schaible *et al.*, 1999). Second, these cells also express cytolytic activities, whereby they lyse infected target cells (Kaufmann, 1999).

$CD4^+$ cells of the Th1 type play a key role in protective immunity against *M. tuberculosis*. This was established, initially, in experimental models of TB (Orme and Collins, 1984). In humans, the strongest evidence for a predominant role of $CD4^+$ T-cells in protective immunity is the increased susceptibility of HIV-infected populations to the development of active TB (Caruso *et al.*, 1999, Orme, 2001). Once macrophages are activated, and the phagocytosed bacteria are processed in the endosomal compartment of the macrophage, peptides will be presented to $CD4^+$ T-cells in association with class II major histocompatibility complex (MHC) molecules on the surface of the macrophage. $CD4^+$ T-cells recognize the complex and become activated, and consequently induce the production of Th1 cytokines, IL-2 and IFN-γ (Flynn *et al.*, 1993).

The role of $CD8^+$ T-cells starts when mycobacteria gain access to the cytoplasmic compartment of macrophages and become associated with MHC-I molecules. This antigen-MHC-I complex can then stimulate $CD8^+$ T-cells to produce cytokines such as IFN-γ and TNF-α to activate macrophages. $CD8^+$ T-cells facilitate the release of intact bacteria entrapped in macrophages. In this way, bacteria can be released from ineffective macrophages and phagocytosed by more efficient cells. Strong evidence from mouse experiments suggests a major role of $CD8^+$ T-cells, in addition to $CD4^+$ T-cells, in protection against TB (Flynn *et al.*, 1992, Lewinsohn *et al.*, 2000, Smith and Dockrell, 2000, Klein and Fox, 2001). Consequently, it is most likely that the different

T-cell populations are required for optimum protection since they have diverse, yet versatile mechanism (Triccas *et al.*, 2002).

The protein and non-protein antigens of *M. tuberculosis* are strong stimuli for induction of cytokine production in human mononuclear phagocytes which most likely affect the outcome of infection at any stage of the *M. tuberculosis* infection (van Crevel *et al.*, 2002). Studies in cytokine production during *M. tuberculosis* infections have shown that several, proinflammatory cytokines, particularly IFN-γ, TNF-α, IL-1, IL-6, IL-12, and IL-18 are produced after the recognition of *M. tuberculosis* by phagocytic cells (Table 1.2). These cytokines act in the autocrine or paracrine networks to influence the function of cells in immunity to *M. tuberculosis* (van Crevel *et al.*, 2002).

Tumor necrosis factor alpha (TNF-α) plays an important role in the host response to infection with *M. tuberculosis*. In mice, TNF-α is essential for formation of tuberculous granulomas which serves to isolate the virulent bacterium and without this cytokine, effective granuloma formation is diminished and bacterial numbers rapidly increase resulting in the death of the mice (Engele *et al.*, 2002).

The importance of IFN-γ in protective immunity against TB derives from two separate lines of evidence. Studies using IFN-γ knockout mice have shown definitively that IFN-γ, a cytokine that activates infected macrophages to kill intracellular bacteria, is vitally important for protection against *M. tuberculosis* (Cooper *et al.*, 1993). Studies of

Table1. 2. Important cytokines during *M. tuberculosis* infection

Cytokine	Function	Source
TNF-α	Granuloma formation. Regulate MΦ function	DC, MΦ, neutrophils, T-cells
IFN-γ	↑ MΦ bactericidal activity	MΦ, neutrophils, T & B cells
IL-12	↑ IFN-γ production by T-cells granuloma formation inducing TH1 response	MΦ
IL-18	Act together with IL-12 to induce IFN-γ	MΦ
IL-1α	MΦ activation T-cell activation	MΦ
IL-4	Th2 response B cell activation	T-cells
IL-10	Suppressive effect on MΦ Inhibits production of TNF-α	T-cells
TGF-β	Suppression of lymphocytes Modulation of proinflammatory cytokine	MΦ, T & B cells

MΦ- macrophages, DC- dendritic cell.

humans who are homozygous for a mutation in IFN-γ receptors show that they are extremely sensitive to fatal mycobacterial infection (Jouanguy *et al.*, 1996, Newport *et al.*, 1996). Individuals who lack the ability to produce IFN-γ, or who cannot respond to it or lack the receptor for it, are susceptible to severe systemic infection with mycobacterial species that do not usually cause significant disease in immunocompetent individuals (Jouanguy *et al.*, 1996, Newport *et al.*, 1996).

Early studies indicated that the purified protein derivative (PPD) of *M. tuberculosis* induces the proinflammatory cytokine IL-1 (Wallis *et al.*, 1996) and TNF-α (Valone *et al.*, 1988). On the other hand the intact *M. tuberculosis*, but not its PPD, was strong in the induction of IL-12 (which stimulates IFN-γ production) in monocytes (Fulton *et al.*, 1998). Both TNF-α and IFN-γ induce microbicidal pathways by producing reactive oxygen and nitrogen intermediaries in phagocytes (Flesch and Kaufmann, 1990).

1.9. Mycobacterial antigens

As previously described, protection against TB is provided through cell mediated immune responses, and the production of IFN-γ by both $CD4^+$ and $CD8^+$ T-cells appears to be essential components of resistance to *M. tuberculosis*. A primary criterion for selecting candidate antigens for vaccine design is their ability to induce protective T-cell responses. Thus, it is required that the major antigens and epitopes of *M. tuberculosis* be identified (see Section 1.11.2). A variety of *M. tuberculosis* antigen preparations obtained from the cell wall, cytosolic fractions and culture filtrates have been reported (Young *et al.*, 1992, Mustafa, 1996, 2002). These antigens have several hundred amino acids, whereas T-cells usually recognize epitopes ranging between 8 and 20 amino acids

presented in the context of MHC molecules. Thus a complete protein antigen may have several T-cell epitopes. To identify the T-cell epitopes mediating protective immunity, several studies have been conducted to identify and characterize the epitopes of *M. tuberculosis* antigens recognized by human T-cells of the Th1 type (Mustafa, 2002).

Although bacteria consist of several thousands of proteins, the range of proteins recognized by antibodies produced in different laboratories was restricted leading to the concept of immunodominance, i.e. that there were certain proteins which were selectively recognized by the immune system. When these antigenic proteins were identified by screening mycobacterial recombinant DNA expression libraries with antibody probes (Young *et al.*, 1985), most of these antigens were found to belong to a group of molecules whose function is to protect the organism from environmental damage (Young *et al.*, 1988). Testing of these antigens with T-cell lines and clones showed that human T-cells recognized most of them (Mustafa, 1988, Oftung *et al.*, 1998, Mustafa *et al.*, 1999). Among these recombinant antigens is the antigen 18-kDa that belongs to the heat shock protein (hsp)18 family (Booth *et al.*, 1988), the 65-kDa which belongs to hsp65 family (Shinnick, 1987) and the 70-kDa which belongs to hsp70 family (Garsia *et al.*, 1989). These antigens were also recognized by human T-cells as natural mycobacterial antigens (Mustafa, 2002). A novel 24 kDa secreted lipoprotein was also identified by direct screening of recombinant DNA libraries with human T-cell clones. Interestingly the T-cell epitopes of this antigen were found to be common to the two most important pathogenic mycobacterial species *M. tuberculosis* and *M. leprae* and was lacking in the vaccine strains of BCG and most environmental mycobacteria (Oftung *et al.*, 1997, Mustafa *et al.*, 1998).

Secreted protein antigens present in culture filtrate preparations have also been studied extensively. These studies showed that these antigens induce CMI responses and are protective against TB in mice and guinea pigs (Andersen, 1994, Horwitz *et al.*, 1995, Roberts *et al.*, 1995). These proteins were separated into discrete fractions according to size by polyacrylamide gel electrophoresis and then tested for T-cell reactivity and proliferation (Abou-zeid *et al.*, 1987). Mouse models of TB infection have shown that T-cells from the memory immune mice secreted large quantities of IFN-γ in response to two fractions of the culture filtrate protein known as the 6-10 kDa proteins and antigen 85 (Ag85) complex (Andersen *et al.*, 1995). Testing of human T-cells with purified antigens of *M. tuberculosis* in proliferation assays and for IFN-γ production showed that the secreted antigens, which include Ag85B, ESAT6, MPT64 and MPB70, were better stimulators of human T-cells compared the antigens of cytosolic origin (Mustafa *et al.*, 1998, Ravn *et al.*, 1999).

Mice immunized with plasmid DNA encoding the Ag85A (DNA-Ag85A) showed a strong T-cell response when restimulated *in vitro* with synthetic 20-mer peptides of the native Ag85. This response was characterized by elevated levels of Th1-type cytokines, IL-2 and IFN-γ. In addition, DNA-Ag85A vaccines conferred protection upon subsequent infection with live mycobacteria (Denis *et al.*, 1998).